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**(54) Title:** METHOD FOR DETECTING CIRCULATING BREAST CANCER CELLS

**(57) Abstract**

The present invention provides an effective method for the detection of circulating breast cancer cells. Based on the observation that one third of breast cancer cells produce prostate-specific antigen (PSA), a preferred embodiment of the clinical test employs a reverse transcriptase (RT) polymerase chain reaction (PCR) assay utilizing oligonucleotide primers specific for nucleic acids encoding PSA. The assay identifies PSA-synthesizing cells from reverse transcribed messenger ribonucleic acid (mRNA). The assay is applied to RNAs extracted from the peripheral blood of breast cancer patients, and can recognize one PSA-expressing metastatic breast cancer cell diluted into one hundred thousand white blood cells. Other amplification methods are also provided to detect PSA mRNA or, alternatively, nucleic acids encoding other antigens expressed by breast cancer cells.

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# METHOD FOR DETECTING CIRCULATING BREAST CANCER CELLS

This application is a continuation-in-part of U.S. Application Serial No. 08/561,952, filed November 22, 1995, the disclosure of which is incorporated herein by reference.

## FIELD OF THE INVENTION

This invention relates to the field of cancer detection. More specifically, the invention provides an effective method for the detection of circulating breast cancer cells in lymph node negative patients.

## BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

The need to identify breast cancer patients who will benefit from adjuvant therapy (chemotherapy), and to spare others the side effects, is spurring researchers to test new prognostic indicators. Adjuvant therapy prolongs the lives of many women with breast cancer. But because doctors cannot be sure which patients' tumors will recur, many patients who do not need treatment receive it nevertheless (1).

Thanks in part to earlier detection, nearly two thirds of newly diagnosed breast cancer cases have no lymph node involvement. Of the 120,000 women every year in this situation, 70-80% can be cured without adjuvant therapy. Identification of the remaining 20-30% of node negative patients who should be given adjuvant therapy remains a dilemma. Among node negative women with small tumors, it is therefore important to ascertain those who will recur, so that they may receive adjuvant therapy,

while sparing those who will not recur the hazards of adjuvant treatment (1).

Tumor size, histologic grading, node involvement, and estrogen receptor status of the tumor are the most widely accepted and widely used indicators employed to assess the probability of tumor recurrence and the need for adjuvant therapy. Other markers, such as tumor epidermal growth factor receptor, tumor c-erbB-2 level, and tumor angiogenesis, are also used (1). However, many of these factors are highly intercorrelated, so the information they provide can be redundant (1). A need exists to identify and develop independent predictors of tumor recurrence. This task is impeded by the complex biological interactions involved in breast cancer, and the concomitant difficulty in predicting which potential markers will provide the best prognostic information.

Prostate cancer is one of the most common cancers in men. Since there is no effective way to prevent this cancer, early diagnosis, treatment and monitoring of these patients is necessary. Recently, a new assay has been employed for detecting circulating metastatic prostate cancer cells. These cells produce a protein, prostate specific antigen (PSA), which is detected by reverse transcription (RT) and polymerase chain reaction (PCR) amplification of the messenger ribonucleic acid (mRNA) encoding the antigen (2-5, 30).

Earlier investigations have suggested that PSA is produced by the prostate gland only, and therefore only expressed in men. Surprisingly, recent studies of tumors of the breast have demonstrated that PSA is detectable in one third of the tumors examined (6-18, 31). PSA levels in breast tumors were examined using a sensitive immunoassay that was capable of detecting PSA at levels of 0.05 $\mu$g/L or higher. The clinical potential of PSA as a prognostic indicator of recurring breast cancer was explored using this same immunological assay

in serum rather than tumor tissue (9). Serum PSA levels were compared in women with and without breast cancer, between women with PSA-positive and PSA-negative breast cancer and between women with breast cancer before and after surgical removal of the tumor. The data showed that in women over 50 years of age, serum PSA levels did *not* vary significantly between normal and breast cancer patients. Furthermore, differences were not observed in pre-surgical and post-surgical serum PSA in women with PSA-positive or PSA-negative breast cancer.

The data summarized above suggest that female serum PSA is not associated with tumor PSA levels; therefore, an immunological PSA assay would not prove clinically efficacious in identifying those women who may have metastatic breast cancer. Moreover, a separate assay, in which PSA mRNA was detected by PCR amplification failed to detect PSA mRNA in a breast cancer cell line (19). Taken together, these results suggest that serum levels of PSA cannot serve as a prognostic indicator of tumor recurrence in node negative women with small tumors. The immunological assay described above appears to be insufficiently sensitive for differentiation in serum of PSA levels between normal and breast cancer patients. The negative result from the breast cancer cell line using a PCR assay further discourages the notion that PSA detection will be useful in a clinical assay to detect circulating breast cancer cells.

## SUMMARY OF THE INVENTION

Even though the studies reported to date would have predicted otherwise, it has been discovered in accordance with the present invention that, surprisingly, a polynucleotide amplification assay for PSA-encoding nucleic acids, particularly mRNA, in a blood sample accurately detects circulating breast cancer cells in approximately one-third of the population tested. This

proportion reflects the proportion of women expected to possess significant amounts of PSA based on immunological analyses of breast tumors. The success of detecting PSA-encoding nucleic acids from blood samples leads also to the expectation that other antigens associated with breast cancer cells can be detected by polynucleotide amplification in a manner similar to the detection of PSA.

In accordance with the present invention, a method of detecting breast cancer micrometastases in a patient is provided. The method comprises obtaining a sample of polynucleotides from a patient's blood, and performing a polynucleotide amplification reaction (such as polymerase chain reaction) on the sample. The polynucleotide amplification reaction is performed using nucleotides that specifically hybridize with sequences present in a predetermined polynucleotide encoding a characteristic determinent of breast cancer cells, under conditions causing amplification of the selected polynucleotide. Thereafter, the presence and quantity of amplification products of the predetermined polynucleotide is detected. The presence and quantity of one or more such amplification products indicates the micrometastases of breast cancer.

As used herein, the term "characteristic determinant" refers to substances such as antigens, haptens and other complex molecules (e.g., carbohydrates, glycoproteins, etc.), which are associated with a cell type of interest. As used herein, the term "predetermined" denotes that a particular characteristic determinant is known to be associated with breast cancer cells. For example, PSA is a characteristic determinant of breast cancer cells in approximately 30% of breast cancers. As used herein, the term "polynucleotide" generally refers to RNA, specifically mRNA, but is also intended to include DNA. Thus, the term "predetermined polynucleotide" refers to a polynucleotide that encodes a

characteristic determinant of breast cancer cells.

As one third of the breast tumors assayed have been found to express PSA, in a preferred embodiment of the invention, the method comprises the detection of mRNA encoding PSA from the blood of a breast cancer patient. An RNA sample is obtained from the patient's blood and the PSA-specific RNA is reverse transcribed and then amplified using a pair of primers which are complementary to separate regions of the PSA DNA. After amplification, the presence or absence of amplified DNA is detected, wherein the presence of amplified DNA indicates micrometastasis of PSA-expressing breast cancer cells.

Various primers for PSA amplification have been described (3, 4, 5, 6, 10, 19, 28, 29); persons skilled in the art would appreciate and could use these and other primers. Also, a nested RT-PCR for a PSA specific membrane antigen has been described (29) and could be used in lieu of the assay delineated above. A digoxigenin-enhanced PSA RT-PCR detection method has been described (5) and could be used as well. Various $^{32}P$ labeled hybridization probes for PSA detection have also been described and used (3, 4, 19, 29).

In a preferred embodiment of the invention, RT-PCR is used to amplify the target polynucleotide. However, because the methods described herein depend on the detection of DNA or mRNA encoding PSA or other breast cancer cell-specific determinant, any polynucleotide amplification method known in the art may be employed.

In yet another preferrred embodiment of the invention the method entails pretreating blood cells from a patient with compounds that induce PSA production. Enhancing production of PSA mRNA prior to the RT-PCR amplification step increases the sensitivity of the assay. Alternatively, steroid hormones capable of inducing PSA production may be administered to the patient for a suitable period prior to obtaining the blood sample. These embodiments of the invention

facilitate detection of PSA expressing breast cancer cells in blood.

Characteristic determinants for breast cancer cells other than PSA may be detected with the above mentioned methodology. These include, but are not limited to, estrogen receptor (ER), progesterone receptor (PR) (20), epidermal growth factor receptor (EGFR) (21), Bcl-2 (22), and erbB-2 protein (23). These antigens are all associated with breast cancer cells, so the polynucleotides encoding these proteins are expected to be detectable with the same assays.

**DETAILED DESCRIPTION OF THE INVENTION**

In accordance with the present invention, a new method for detecting metastases in breast cancer patients is disclosed. Based on a report that a third of breast cancers produce prostate specific antigen (PSA), the present inventor surmised that the reverse transcriptase-polymerase chain reaction for PSA, used for detecting metastases in prostate cancer, might also be used for detecting breast cancer metastases. As described in greater detail in Example 1, clinical experiments with several breast cancer patients revealed that the PSA assay detects circulating breast cancer cells in approximately one third of the population tested. This assay is expected to be useful as an additional independent predictor of tumor recurrence in node negative breast cancer patients.

An exemplary embodiment of the clinical test employs an enhanced reverse transcriptase (RT) polymerase chain reaction (PCR) assay utilizing oligonucleotide primers specific for nucleic acids encoding the human prostate-specific antigen (PSA). This assay identifies PSA-synthesizing cells from reverse transcribed mRNA. The assay is applied to RNAs extracted from the peripheral blood of breast cancer patients. The RT-PCR assay for PSA can recognize one PSA-expressing metastatic

breast cancer cell diluted into one hundred thousand white blood cells. The sensitivity of the assay may also be enhanced by the addition of digoxigenin-modified nucleotides to the PCR reaction.

In another embodiment of the invention, the sensitivity of the assay is enhanced by pretreating the blood sample to be tested with steroid hormones. It has been discovered that androgens, progestins, mineralocorticosteroids, glucocorticosteroids and antiestrogens upregulate PSA production in receptor positive breast carcinoma cell lines, T-47D and MCF-7. In the alternative, steroid hormones may also be administered to a patient for a suitable period before obtaining the blood sample to be tested.

Although polynucleotide amplification by the polymerase chain reaction is described and exemplified herein, it will be appreciated by persons skilled in the art that a variety of other methods for polynucleotide amplification of a target DNA or mRNA may be utilized in the present invention. These include, but are not limited to: 1) targeted polynucleotide amplification methods such as self-sustained sequence replication (3SR) and strand-displacement amplification (SDA); 2) methods based on amplification of a signal attached to the target polynucleotide, such as "branched chain" DNA amplification (Chiron Corp.); 3) methods based on amplification of probe or primer DNA, such as ligase chain reaction (LCR) and QB replicase amplification (QBR); and 4) various other methods, such as ligation activated transcription (LAT), nucleic acid sequence-based amplification (NASBA), repair chain reaction (RCR) and cycling probe reaction (CPR). For a review of these methods, see pp. 2-7 of <u>The Genesis Report</u>, DX, Vol. 3, No. 4, Feb. 1994; Genesis Group, Montclair, NJ).

In accordance with the present invention, it is also noted that several other specific characteristic determinants are present on circulating breast cancer

cells. In another embodiment of the present invention, these determinants will be assayed as well. Two thirds of breast cancer cells produce estrogen receptor (ER) protein (20), and so the same assay, amplifying ER mRNA rather than PSA mRNA, is expected to be able to detect breast cancer cells producing ER. Since breast cancer cells produce progesterone receptor (PR) (20), epidermal growth factor receptor (EGFR) (21), Bcl-2 (22), and erbB-2 protein (23), the mRNAs encoding these proteins should be detectable with the same assay. The nucleotide sequences for the primers for ER (24), PR (24), EGFR (25), Bcl-2 (26) and erbB-2 (27) are well known; these primers have been synthesized and are readily available.

The availability of nucleotide sequence information enables preparation of isolated nucleic acid molecules of the invention by oligonucleotide synthesis. These oligonucleotides are designed to hybridize specifically with sequences on the target polynucleotide. As used herein, a "specifically hybridizing" oligonucleotide is one of sufficient complementarity to a specified region of the target polynucleotide (i.e., the predetermined polynucleotide) to hybridize substantially exclusively with that region under standard hybridization conditions (i.e., conditions normally used for a given polynucleotide amplification reation). Fully complementary oligonucleotides are preferred. Depending on design, these oligonucleotides may be utilized in a variety of polynucleotide amplification reactions, for example, primers for polymerase chain reaction, oligonucleotides that bind contiguous stretches of the DNA encoding the targeted determinant, for ligase chain reaction, etc. Other uses for oligonucleotides in targeted polynucleotide amplification reactions will be apparent to those skilled in the art.

Synthetic oligonucleotides may be prepared by standard methods, such as the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or

similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). General procedures to synthesize and purify oligonucleotide, are set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989).

The methodology described herein can also be used to detect micrometastases in regional lymph nodes, as a breast cancer screening technique, or for evaluating the results of adjuvant therapy (hormone therapy, chemotherapy, etc.). Moreover, the PSA mRNA assay may be used to detect circulating metastatic cells in patients with other tumor types that express PSA.

The following non-limiting example is provided to describe the invention in greater detail.

## Example 1

**RNA Preparation:**

Six to eight milliliters of peripheral venous blood was obtained using heparinized tubes and placed immediately on ice. The whole blood was then subjected to a gradient isolation of nucleated cells using Ficoll (Accurate Chemical and Scientific Corp, Westbury, NY) (4). The mononuclear cell layer was aspirated, re-diluted in phosphate-buffered saline, and then centrifuged as previously described (4). After the supernatant was discarded, the pellet was stored at -70°C or used directly for RNA extraction. After adding 2 mL of RNAzol B (Biotecx Laboratories, Houston, TX) and 0.2 mL of chloroform to the pellet, the preparation was mixed vigorously and put on ice for 5 minutes. The suspension was then centrifuged at 12,000 x g (4°C) for 15 minutes. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of isopropanol. The samples were then put at -20°C for at least 2 hours. This was followed by centrifugation at 12,000 x g (4°C) for 15 minutes. After the supernatant was discarded, the RNA pellet was washed with 100% ethanol and subsequently centrifuged at 12,000 x g (4°C) for 15 minutes. This washing step was repeated using 75% ethanol. The dry RNA pellet was finally dissolved in 50 $\mu$L of diethylpyrocarbonate-treated water.

**RT-PCR and Primers:**

Approximately 400 ng of RNA from each sample were subjected to an RT-PCR using primers PSA3' and PSA5' as previously described (5). The 18 base pair primers were designed to span across three exons; from exon 3 and extending into exon 5 with the following sequences:

PSA3': 5'-CACAGACACCCCATCCTATC-3' (Sequence I.D. No. 1)

PSA5': 5'-GATGACTCCAGCCACGACCT-3' (Sequence I.D. No. 2)

The entire PCR products were run on a 2% ethidium bromide-stained agarose gel, then transferred to a nylon membrane using the Oncor Probe Tech 2 system (Oncor, Gaithersburg, MD). The membranes were pre-hybridized at 42°C using Hybrisol I (Oncor) as a pre-hybridization mixture. Hybridization was performed at 42°C for 16 hours with a 32p end-labeled probe internal to the PCR primers: R2: 5'-CTACGCCTCAGGCTGGGGCAGCATTGAACCAGAGGAGTTCTTGACC-3' (Sequence I.D. No. 3). This was followed by washes of increasing stringency (final, 52° to 54°C) with 0.1% sodium dodecyl sulfate (SDS)/0.1% sodium chloride-sodium citrate. The blots were exposed to X-OMAT films (Eastman Kodak, Rochester, NY) at -70°C for 48 hours using intensifying screens.

**Results**

Forty-six peripheral blood samples from women with breast cancer were tested. In five of twelve samples from women with disease limited to the breast, PSA mRNA was amplified and detected by the above methods. In four of sixteen samples from women whose disease had spread to the axillary lymph nodes, PSA mRNA was amplified and detected. In samples from eight of eighteen women who had distant metastases to bone or brain, PSA mRNA was amplified and detected. Thus, 17 out of 46, or approximately one-third, of the patients tested, could be identified as having circulating metastatic breast cancer cells. In contrast, in samples from 20 female controls (2) and six female controls (3) there was no PSA mRNA amplification or detection. These results demonstrate that the above-described assay can be employed in a clinical assay as a prognostic indicator of tumor recurrence. Additionally, the several other cell markers present on circulating breast cancer cells should likewise be useful in clinical assays for node negative breast cancer patients.

**EXAMPLE II**

Yu et al. (12) have demonstrated that the steroid hormone receptor-positive breast carcinoma cell lines T-47D and MCF-7 can be induced to produce prostate specific antigen (PSA) by the following steroids: androgens, progestins, mineralocorticosteroids, glucocorticosteroids, and antiestrogens. Estrogens failed to induce such stimulation in both cell lines and, in addition, Yu et al. were able to block the induction by androgens in the cell line T-47D. PSA can also be induced in prostate cancer cell lines by incubation with androgen (32). These data indicate that the sensitivity of the PSA-RT-PCR assay for detecting circulating breast tumor cells or prostate tumor cells can be increased by incubation of the blood specimen or white blood cells with the following steroids: androgens, progestins, mineralocorticosteroids, glucocorticosteroids, or antiestrogens before the PSA RT-PCR is performed.

To stimulate production of PSA-mRNA, the steroids are dissolved to make stock solutions of $10^{-2}$ to $10^{-3}$ M in absolute ethanol. White blood cells from a patient sample are aliquoted in 24 well tissue culture plates and 2$\mu$L of each steroid, dissolved in absolute ethanol, added to each well and incubated at 37.6° from one hour to eight days. Because the assay is performed on a multiwell plate, samples from several patients may be tested simultaneously.

Alternatively, androgens, progestins, mineralocorticosteroids, glucocorticosteroids, or antiestrogens could be administered directly to the patient and blood drawn for analysis after 24-48 hours.

Suitable androgens for the above assay are testosterone, dihydroandrosterone, androsterone,

R1881 (Methyltrienolone), R5020, dihydrotestosterone.

Suitable glucocorticoids are corticosterone, hydrocortisone, betamethasone-17-valerate, dexamethasone and triamcinolone acetonide.

Progestins such as progesterone, norethynodrel, norethindrone, norgestrel, depo-provera, norgestimate may also be used.

A mineralocorticoid that could be used is aldosterone.

Cyproterone acetate, an antiandrogen/progestin may also be utilized. Other antiandrogens that may prove to be useful are RU56,187, mifepristone, cortexolone, spironolactone. An antiestrogen that may stimulate PSA-mRNA production is tamoxifen.

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While certain embodiments of the present invention have been described and exemplified above as preferred embodiments, the invention is not limited to those embodiments, but is capable of variation and modification within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lehrer, Steven

(ii) TITLE OF INVENTION: Method for Detecting Circulating Breast Cancer Cells

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACAGACACC CCATCCTATC 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATGACTCCA GCCACGACCT 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTACGCCTCA GGCTGGGGCA GCATTGAACC AGAGGAGTTC TTGACC
46

**What is claimed is:**

1. A method of detecting circulating breast cancer micrometastases in a patient, comprising the steps of:

obtaining a sample of polynucleotides from a patient's blood;

performing a polynucleotide amplification reaction on said sample using oligonucleotides that specifically hybridize with sequences present in a predetermined polynucleotide encoding a characteristic determinant of breast cancer cells, under conditions causing said amplification of said polynucleotide; and

detecting amplification products of said predetermined polynucleotide, the presence thereof being indicative of said circulating breast cancer micrometastases.

2. The method of claim 1, wherein said predetermined polynucleotide is RNA.

3. The method of claim 1, wherein said characteristic determinant of breast cancer cells is selected from the group consisting of prostate-specific antigen (PSA), estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor (EGFR), Bcl-2, and erbB-2 protein.

4. The method of claim 1, wherein said polynucleotide amplification reaction is a polymerase chain reaction and said oligonucleotides comprise a pair of primers that specifically hybridize with sequences comprising separate regions of said predetermined polynucleotide.

5. The method of claim 4, wherein said

predetermined polynucleotide is RNA and said polynucleotide amplification reaction is a reverse transcriptase polymerase chain reaction.

6. The method of claim 4, wherein said polymerase chain reaction comprises digoxigenin-modified nucleotide triphosphates.

7. The method of claim 3, wherein said predetermined polynucleotide encodes PSA.

8. The method of claim 7, wherein said polynucleotide amplification reaction is a polymerase chain reaction and said oligonucleotides comprise a pair of primers that specifically hybridize with sequences comprising separate regions of said PSA-encoding polynucleotide.

9. The method of claim 1, wherein said sample of polynucleotides is obtained from a mononucleated cell layer prepared from said blood.

10. A method of detecting circulating breast cancer micrometastases in a patient, comprising the steps of:

obtaining a sample of polynucleotides from a patient's blood;

performing a polynucleotide amplification reaction on said sample using oligonucleotides that specifically hybridize with sequences present in a polynucleotide encoding PSA, under conditions causing said amplification of said PSA-encoding polynucleotide; and

detecting amplification products of said PSA-encoding polynucleotide, the presence thereof being indicative of said circulating breast cancer micrometastases.

11. The method of claim 10, wherein said PSA-encoding polynucleotide is RNA.

12. The method of claim 10, wherein said polynucleotide amplification reaction is a polymerase chain reaction and said oligonucleotides comprise a pair of primers that specifically hybridize with sequences comprising separate regions of said PSA-encoding polynucleotide.

13. The method of claim 12, wherein said primers have the sequences
5'-CACAGACACCCCATCCTATC-3' (Sequence I.D. No. 1); and
5'-GATGACTCCAGCCACGACCT-3' (Sequence I.D. No. 2).

14. The method of claim 12, wherein said predetermined polynucleotide is RNA and said polynucleotide amplification reaction is a reverse transcriptase polymerase chain reaction.

15. The method of claim 12, wherein said polymerase chain reaction comprises digoxigenin-modified nucleotide triphosphates.

16. The method of claim 10, wherein said sample of polynucleotides is obtained from a mononucleated cell layer prepared from said blood.

17. A method of detecting circulating breast cancer micrometastases in a patient, comprising the steps of:

obtaining a sample of RNA from a said patient's blood;

amplifying said RNA by reverse transcriptase polymerase chain reaction using a pair of primers which are complementary to separate regions of an RNA molecule encoding PSA; and

detecting said amplified PSA-encoding RNA, if present in said sample, the presence thereof being indicative of said circulating breast cancer micrometastases.

18. The method of claim 17, wherein said primers have the sequences 5'-CACAGACACCCCATCCTATC-3' (Sequence I.D. No. 1); and 5'-GATGACTCCAGCCACGACCT-3' (Sequence I.D. No. 2).

19. A method of detecting circulating breast cancer cells in a patient, comprising the steps of:

treating said patient's blood with a compound inducing PSA production;

obtaining a sample of polynucleotides from said treated blood sample;

performing a polynucleotide amplification reaction on said sample using oligonucleotides that specifically hybridize with sequences present in a polynucleotide encoding PSA, under conditions causing said amplification of said PSA-encoding polynucleotide; and

detecting amplification products of said PSA-encoding polynucleotide, the presence of said products being indicative of said circulating breast cancer micrometastases.

20. A method according to claim 19, wherein said PSA inducing compound is selected from the group comprising androgens, progestins, mineralocortocoids, glucocortocoids, or antiestrogens.

21. A method according to claim 20, wherein said PSA inducing compound is administered to a patient prior to obtaining said blood sample.

22. A method according to claim 20, wherein a withdrawn sample of blood is treated with said PSA inducing compound.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10647

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04
US CL :435/6, 91.2; 536/24.33
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, EMBASE, BIOSIS, MEDLINE, INPADOC, DISSABS, USPATFULL
search terms: PSA, prostate specific antigen, breast cancer, amplification, hybridization, PCR

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | SMITH ET AL. Prostate-specific Antigen Messenger RNA Is Expressed in Non-Prostate Cells: Implications for Detection of Micrometastases. Cancer Research. June 1995, Vol. 55, pages 2640-2644. See page 2640, column 2 through page 2642, column 1. | 1-5, 7-12, 14, 16, 17, 19-22<br>------<br>6, 13, 15, 18 |
| Y | KATZ ET AL. Molecular Staging of Prostate Cancer with the Use of an Enhanced Reverse Transcriptase-PCR Assay. Urology. June 1994, Vol. 43, Number 6, pages 765-775. See abstract and page 768. | 1-22 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 AUGUST 1996 | 03 SEP 1996 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>AMY ATZEL |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| --- | --- | --- |
| Y | MONNE ET AL. Molecular Characterization of Prostate-specific Antigen Messenger RNA Expressed in Breast Tumors. Cancer Research. 15 December 1994, Vol. 54, pages 6344-6347. See abstract and page 6347, last paragraph. | 1-22 |
| Y | US 5,344,757 A (HOLTKE ET AL.) 06 September 1994. See examples 17-29 in columns 14-24. | 6, 15 |